Development of animal-free, protein-free and chemically-defined media for NS0 cell culture

Jinyou Zhang* and David Robinson

*Bioprocess R&D, Merck Research Laboratories, PO Box 2000, Rahway, NJ 07065, USA; *Author for correspondence (e-mail: jinyou_zhang@merck.com; phone: +1-732-5942915; fax: +1-732-594-2915)*

Received 29 June 2005; accepted in revised form 28 September 2005

Key words: Animal-free, Chemically-defined, Glutamine synthetase, Growth, GS-NS0, Medium development, Monoclonal antibody, NS0, Production, Protein-free, Serum-free, Shake-flask

Abstract

There has been a recent boom of monoclonal antibodies on the market, and a significant portion of them were produced by NS0 cell lines. As regulations become more stringent in ensuring production processes are free of potential contamination by adventitious agents, it is highly desirable to further develop serum-free media into ones that do not contain any components of animal origin, or 'animal-free media'. Using a shake-flask batch culture system, recombinant proteins (human albumin and human insulin) and synthetic compounds (tropolone and ferric ammonium citrate) were identified to be capable of replacing the animal-sourced proteins commonly found in serum-free media for NS0 cell culture, namely bovine albumin, insulin and transferrin. The cholesterol requirement of NS0 cells was satisfied by the use of a commercially available non-proteinaceous, non-animal sourced cholesterol/fatty acid mix in place of bovine lipoproteins, which in effect also eliminated the need for recombinant albumin. In the animal-free medium thus formulated, NS0 cell lines, either the host or recombinant constructs, were all able to grow in batch culture to $1\sim 3\times 10^6$ viable cells/ml for multiple passages, with no requirement for gradual adaptation even when seeded from 10% serum-containing cultures. It was surprising to observe that the recombinant insulin was essentially ineffective as sodium salt compared to its zinc salt. Studies showed that the zinc deficiency in the former resulted in a rapid decline of cell viabilities. Supplementation of zinc ions greatly improved growth, and even led to the total replacement of recombinant insulin and hence the formulation of a protein-free medium. When the cell lines were adapted to cholesterol-independent growth which eliminated the need for any lipid source, a completely chemically-defined animal-free medium was formulated. In all cases, antibody production by various GS-NS0 constructs in animal-free media was stable for multiple passages and at least similar to the original serum-free medium containing the animal-sourced proteins. The medium also served well for cryopreservation of NS0 cells in the absence of serum.

Introduction

Owing to their medical benefit and technology advancement, nineteen monoclonal antibody (MAb) based therapeutic agents have gained FDA approval and almost 200 more are currently in

various stages of clinical development (Das and Morror 2004; FDA 2004). Most of the 17 MAbs approved since 1997 were in the oncology and immunology therapeutic areas that address the unmet medical needs. At least four of these were produced in recombinant NS0 cells (Table 1),

demonstrating the attractiveness of this cell line for commercial production of biopharmaceutical proteins.

NS0 is a nonsecreting subclone of the NS-1 mouse myeloma cell line (Kohler and Milstein 1976; Galfre and Milstein 1981). Due to their hardy, suspension growth characteristics and their well-equipped machinery for producing and secreting immunoglobulin proteins, NS0-derived recombinant constructs are commonly employed for MAb expression (Birch and Froud 1994; Barnes et al. 2000). In addition, NS0 cells are well-suited for use of the glutamine synthetase (GS) expression system because they lack endogenous GS activity, thus a plasmid containing both GS and the antibody gene of interest can be constructed to allow easy isolation of MAb-secreting transfectants in glutamine-free medium (Bebbington et al. 1992). Through process optimization, MAb production from the GS-NS0 system has reached over 2.5 g/l in fed-batch reactor cultures (Zhou et al. 1997; Varma et al. 2003; Schmelzer et al. 2004).

As more MAbs are being employed as therapeutic agents, their production methods are coming under increasing regulatory scrutiny. Among the concerns is the presence of animal-derived components in the media employed to grow mammalian cells used for antibody production, because animal-derived substances can potentially introduce contaminants (adventitious agents) into the process and thus potentially into the final

product. Regulations have become more stringent in order to ensure that the biopharmaceutical production process is free of potential contamination by adventitious agents such as that causing Bovine Spongiform Encephalopathy (BSE). For example, European regulations specifically require that non-animal alternatives be used where available, and demand that 'manufacturers shall always justify the use of materials of animal origin' (EMEA 2002). There are clearly risks associated with using animal sourced materials to produce therapeutic compounds, as these materials could be a source for prion protein transmission (Galbraith 2002) and viral contamination (Merten 2002). Although the commonly used serum-free (SF) media no longer contain animal serum as an additive, they still often contain animal-derived components, particularly bovine proteins. Because any animal-derived proteins used could carry a theoretical risk of introducing prions (Robwer 1996) or other adventitious agents (Hellman et al. 1996), it is highly desirable to develop animal protein-free (APF) media for cell culture processes (Merten 1999). A protein-free (PF) medium may minimize even the indirect risks, such as those potentially from fermentation raw materials used during production of the recombinant proteins to be employed in APF media. PF medium also benefits the downstream processing of protein antibodies. Ultimately for these purposes, the chemically-defined (CD) media are protein-free as

Table 1. Recently marketed monoclonal antibodies and their production host cells.

Product	Manufacturer	Launch	Indication	Production Host
Rituxan	Idec	1997	Non-Hodgkin's lymphoma	CHO
Zenapax	Roche	1997	Organ rejection	NS0
Simulect	Novartis	1998	Organ rejection	SP2/0
Remicade	Centocor	1998	Rheumatoid arthritis	NS0
Herceptin	Genentech	1998	Breast cancer	CHO
Synagis	MedImmune	1998	Respiratory syncytial virus	NS0
Enbrel	Immunex	1998	Rheumatoid arthritis	CHO
Mylotarg	Wyeth	2000	Acute myelogenous leukemia	NS0
Campath	Millennium	2001	Lymphocytic leukaemia	CHO
Zevalin	Idec	2002	Non-Hodgkin's lymphoma	CHO
Humira	Abbot	2002	Rheumatoid arthritis	HEK293
Xolair	Genentech	2003	Asthma	CHO
Bexxar	Corixa/GSK	2003	Non-Hodgkin's lymphoma	Hybridoma
Raptiva	Xoma/Genen.	2003	Plaque psoriasis	CHO
Erbixux	Imclone/BMS	2004	Cancer (colorectal)	SP2/0
Avastin	Genentech	2004	Cancer (colon or rectum)	CHO
Tysabri	Biogen Idec	2004	Multiple sclerosis	Murine myeloma

well as devoid of any undefined components. Although APF, PF and CD media may possibly still contain animal-originated small molecule components, the formulations can be readily made entirely animal-free (AF). This is because from the cell culture performance point of view, the replacement of these small compounds in media, such as certain amino acids, is typically straightforward using non-animal sourced counterparts currently available (Jayme 1999), which are derived from recombinant fermentation, synthetic or plant sources.

Cell culture medium is usually developed by selecting a well-established and chemically-defined basal formulation, and supplementing it with additives specific to the targeted cell line or recombinant construct. The development process is usually iterative until the desired growth and production levels are achieved at large scale, together with satisfactory product purification and product quality. The commonly used strategies for medium formulation and optimization include literature survey which identifies possible growth requirements based on related cell lines or constructs, and component swapping which improves performance in a simple and effective manner. Other approaches are also used, such as material balance to estimate minimum nutritional needs based on cell compositions and available intracellular stoichiometry (Xie and Wang 1994, 1997), spent medium analysis to identify and fortify limiting nutrients (Jayme 1991; Gambhir et al. 1999), statistically-designed experimentation to gather rich data including component interactions using relatively few experiments (Castro et al. 1992; Tsai et al. 2003). These latter few methods have been used less frequently in cell culture medium development because of the incomplete understanding of metabolic pathways, and the huge numbers of medium components in addition to their high complexity. More recently, the genomic and proteomic approach has been employed to develop and optimize cell culture media (Allison et al. 2004), which allows targeted identification by microarray of ligand compounds for the specific receptors expressed by the cells, thus avoiding random testing. However, since too many receptors are detected, its applicability will practically depend on how well one can determine and manipulate the physiologically important pathways in a given culture system.

Using the above approaches, researchers have published several papers on the development of serum-free media for NS0 culture processes (Brown et al. 1992; Buser et al. 1994; Robinson et al., 1995; Dempsey et al. 2003). Break-through has also been in using these media for cell freezing and preservation (Merten et al. 1995). But to preserve the beneficial functions of serum, these media still contained a number of bovine-derived large constituents, such as albumin, insulin, transferrin, and lipids. Significant progress was recently made in replacing animal constituents with non-animal substitutes in general (Jayme 1999), and in developing protein-free and chemically-defined media specifically for NS0 growth and recombinant protein production (Keen and Hale 1996; Gorfien et al. 2000; Mainwaring et al. 2002; Talley et al. 2003; Whitford et al. 2004). However, due to the competitive nature of the industry, detailed information on development and compositions is proprietary and mostly unavailable.

In this communication, we describe our development of animal-free, protein-free and even chemically-defined media from a published serum-free composition for NS0 wild-type cells and recombinant constructs.

Materials and methods

Cell lines

Wild type (host) as well as recombinant NS0 murine myeloma cells transfected with the GS expression vector system (licensed from Lonza Biologics) were used in this study. The NS0 wild-type cell line (NS0-WT) was obtained from the European Collection of Cell Culture (ECACC 85110503). NS0 lacks endogenous glutamine synthetase which catalyzes the formation of glutamine from glutamate and ammonia, enabling the selection and the culture of GS-NS0 recombinants in glutamine-free media (Bebbington et al. 1992). GS-NS0 Cell Line I secreted a model IgG1 MAb protein, while Cell Line II (licensed from Lonza Biologics) and III both expressed a model IgG4 MAb protein. NS0-WT and Cell Line I were also adapted to cholesterol-independent growth, and used to develop chemically-defined media. All cell lines were maintained in cryo-vials until use, and

when needed the subsequent passage numbers were indicated in respective figures.

Materials

The glutamine-free Iscove's Modified Dulbecco's Medium (IMDM), amino acids, nucleosides, ethanolamine, β -mercaptoethanol, tropolone, ferric ammonium citrate, the sodium salt of recombinant human insulin (rHI), and zinc chloride were from Sigma-Aldrich, all at cell-culture grade. The zinc salt of rHI was from Roche Diagnostics, recombinant human albumin (rHA) from Delta Biotechnology, Pluronic F68, glutamine and Cholesterol Lipids Concentrate (CLC) from Invitrogen, bovine insulin and Excyte VLE (bovine lipoproteins) from Serologicals, Albumax (bovine serum albumin) from GibcoBRL, bovine transferrin from Interger, Fetal Bovine Serum (FBS) from Hyclone, and GS Supplements from JRH.

Media

For serum-containing medium, 10% FBS was added to IMDM basal medium along with 1 $\times$ GS Supplements. It should be noted that in this communication, as used by the suppliers or for proprietary reason, the concentration of a medium component was usually expressed in a relative

term, with 1 $\times$ as the normal level used in the composition. For other media, various additives were added as specified to IMDM to formulate the complete media. Where needed, the additives (Table 2) were prepared as stock solutions and stored at 4 °C (glutamine at -20 °C). All complete media were stored for ≤ 2 months at 4 °C. Before use, the media were warmed to 37 °C in a water bath. For GS-NS0 cell lines, no glutamine was added, but glutamate and arginine were still present.

Cell culture methods

Following vial-thaw, cells were seeded either into 10% FBS medium or directly into other specified media. For shake-flask culture, an orbital shaker (Cole Palmer 51300-series) was placed in a Forma water-jacketed CO₂ incubator maintained at 37 °C with constant supply of 5% CO₂ and about 85% humidity. Culture growth volume was usually 15 ml in a 125-ml Corning Erlenmeyer flask (polycarbonate, #430421, with vented cap), and the rotation speed was 50–75 rpm. Under these conditions, the evaporation rate was estimated to be less than 1% of the growth volume per day. An exponentially-growing culture (Day 3–4) was normally used as the inoculum, at 1–1.5 $\times 10^5$ viable cells/ml. Additional conditions were specified in the individual figures. Only batch culture

Table 2. Development of animal-free media from serum-free medium for NS0 cells.

Serum-free medium (SFII) (Robinson et al. 1995)	Animal-free medium (AF)	Protein-free medium (PF)	Chemically-defined medium (CD)
Cholesterol-dependent IMDM basal medium	Cholesterol-dependent IMDM basal medium	Cholesterol-dependent IMDM basal medium	Cholesterol-independent IMDM basal medium
Amino acids	Amino acids	Amino acids	Amino acids
Nucleosides	Nucleosides	Nucleosides	Nucleosides
β -mercaptoethanol	β -mercaptoethanol	β -mercaptoethanol	β -mercaptoethanol
Ethanolamine	Ethanolamine	Ethanolamine	Ethanolamine
Pluronic F68	Pluronic F68	Pluronic F68	Pluronic F68
Glutamine ^a	Glutamine ^a	Glutamine ^a	Glutamine ^a
Bovine transferrin	Tropolone/Fe ammon. citrate	Tropolone/Fe ammon. citrate	Tropolone/Fe ammon. citrate
Bovine insulin	Rec. human insulin (zinc salt)	Zinc chloride	Zinc chloride
BSA (Albumax)	Recombinant human albumin		
Bovine lipoprotein (Excyte)	Cholesterol lipid concentrate	Cholesterol lipid concentrate	

^aNot needed for GS-NS0 cell lines.

results from shake-flasks are reported in this communication.

Analytical methods

At time intervals specified in individual figures, a half milliliter of cell suspension was taken from each flask for cell growth and nutrient/metabolite analyses. At Day-7, a sample was collected and stored at 4 °C until assay of IgG proteins.

Viable cell density (VCD, expressed as viable cells/ml or vc/ml) and viability were determined by hemocytometer counts after trypan-blue staining. Glucose, lactate, glutamine and glutamate concentrations in the culture broth were measured on YSI systems (YSI Life Sciences). The concentration of IgG1 antibody protein was assayed by Protein A affinity chromatography method (Zhou et al. 1997), while that of IgG4 was determined by either BIAcore mass assay method (Mullett et al. 2000) or the Protein A method. Both IgG1 and IgG4 concentrations are expressed as unit/ml for proprietary reasons.

Results and discussion

Substitution of animal-derived proteins in serum-free medium with synthetic or recombinant components

A serum-free medium (SFII) composed of IMDM and various additives was first developed and published earlier for recombinant GS-NS0 constructs (Robinson et al. 1995). There were four components from animal origin, namely bovine transferrin, bovine insulin, bovine serum albumin (Albumax), and bovine lipoproteins (Excyte). Based on a review of the literature (Kovar and

Franek 1987; Metcalfe et al. 1994; Cotter and Al-Rubeai 1995; Gorfien et al. 1998) and some preliminary studies, the non-animal replacements for these proteinaceous components were identified, as summarized in Table 2, which also includes the substitutes developed later during protein-free or chemically-defined medium development.

By using the synthetic iron carrier tropolone (Metcalfe et al. 1994; Field 2003) plus ferric ammonium citrate as the iron source (Kovar and Franek 1987), rHI, and rHA to swap for bovine-derived transferrin, insulin, and Albumax, respectively and individually, GS-NS0 Cell Line I grew comparably well with viabilities similar to SFII medium (Table 3). Its growth was apparently very sensitive to the level of tropolone, as doubled concentration led to a sharp drop of maximum viable cell density from 1.2 to 0.2×10^6 vc/ml. While increasing concentrations of rHA up to $2 \times$ supported better growth (Table 3), a low level ($0.5 \times$) was selected due to the concern on the cost of this recombinant protein and the consideration to minimize the amount of exogenous protein in the media.

These replacements were then combined into a medium to compare with the original SFII medium for multiple passage growth of Cell Line I (Figure 1). It was clear from the comparison that the combination of these non-animal-sourced components worked well together, achieving 1.5 – 2.5×10^6 vc/ml which was higher than the 1 – 2×10^6 by the use of their bovine counterparts. It can also be seen that no adaptation was needed for growth in the substituted medium after seeding directly from an SFII culture.

Media containing even lower levels of rHA in the presence of tropolone/ferric ammonium citrate and rHI were evaluated. It was eventually demonstrated that for Cell Line I, media containing $0.1 \times$ rHA supported growth and IgG1 titers

Table 3. Individual replacement of animal proteins in SFII medium to formulate animal protein-free medium for GS-NS0 Cell Line I.

Non-animal replacement (bovine protein replaced)	SFII control	Tropolone/FAC (transferrin)			rHI (insulin)			rHA (albumin)		
Concentration	–	0.5×	1×	2×	0.5×	1×	2×	0.5×	1×	2×
Viable cells/ml ($\times 10^6$)	1.4	1.2	1.3	0.2	1.0	0.9	1.4	1.0	1.3	1.7
Viability (%)	88	84	83	46	65	71	88	86	85	90

The seed cells for all experiments were previously grown in SFII medium. Bovine proteins were replaced one by one with non-animal components, at specified concentrations (expressed as fold of an arbitrary base level). Tropolone and FAC (ferric ammonium citrate) were always used at a 15:1 w/w ratio. In each case, the medium still contained Excyte as the lipid source. The viable cell densities and viabilities were the peak values from 7-day batch shake-flask cultures at the first passage.

Figure 1. Replacement of animal proteins in SFII medium to formulate animal protein-free medium for GS-NS0 Cell Line I. $\square$ SFII medium; $\triangle$ animal protein-free medium (contained 1 $\times$ tropolone/ferric ammonium citrate, 2 $\times$ rHI and 0.5 $\times$ rHA to replace bovine transferrin, insulin and albumin in SFII medium, respectively). Both media contained 0.5% (v/v) Excyte as lipid source. Graphs show growth profiles of both cultures during multiple passages in shake-flasks.

comparable to media containing 0.5 $\times$ rHA (data not shown).

Replacement of bovine lipids with non-animal lipid mix and elimination of rHA

NS0 cells are auxotrophic for cholesterol (Sato et al. 1988; Keen and Steward 1995). While the medium described above were free from

animal-derived insulin, transferrin and albumin, they still contained Excyte, a bovine lipoprotein product, as the lipid source for NS0 cultures. Its animal origin and undefined nature necessitated the search for alternatives. Among others tested, the commercially available non-animal cholesterol/fatty acids mix, CLC, was identified as an appropriate replacement. As shown in Figure 2, NS0-WT cells, seeded directly from the Excyte-containing SFII culture, grew immediately in the

Figure 2. Replacement of bovine-derived Excyte with non-animal lipids in animal protein-free medium for NS0-WT cells. $\square$ SFII medium with 0.5% (v/v) Excyte; $\triangle$ APF medium with 0.2 $\times$ Cholesterol Lipid Concentrate or CLC (contained no rHA nor Excyte). Graphs show growth profiles during multiple passages in shake-flasks for both cultures.

Figure 3. Direct growth without adaptation for serum-requiring NS0-WT cells in animal-free medium from 10% FBS-growing or frozen culture. ○ 10% FBS in IMDM medium, seeded from vial-thaw; □ AF medium, seeded from 10% FBS; △ AF medium, seeded from vial-thaw. AF medium contained 0.3×CLC lipids, but no rHA nor Excyte. Graphs show growth profiles during multiple passages in shake-flasks for both cultures.

now animal protein-free medium with CLC as the sole lipid source, and the growth was comparable with SFII medium for multiple passages. Surprisingly, in this CLC-containing APF medium, the complete omission of rHA did not impact culture

performance (Figure 2), suggesting that rHA might have been mainly functioning in the Excyte-containing medium as a lipid carrier, which became obsolete due to the presence of an alternative carrier (β -methyl-cyclodextrin) in CLC.

Figure 4. No adaptation from 10% FBS to animal-free medium by serum-requiring GS-NS0 Cell Line II and its comparable performance in SFII and AF media. △ and ▲ SFII medium (contained Excyte); □ and ■ AF medium (contained 0.3×CLC, but no Excyte nor rHA). Open symbols represent growth, and closed symbols production. The IgG4 titers shown were the day-7 results at various passage numbers. Graphs show growth and production profiles during multiple passages in shake-flasks for both cultures. Note that in this experiment, growth was measured only up to Day-4 for each passage, while titers were assayed only for the Day-7 samples.

As pointed out earlier, the substitution of the remaining animal-sourced small molecule components was expected to be straightforward and not impacting cell culture performance. Therefore from this stage on, the formulation developed thus far was directly referred as animal-free or AF medium, and was used in the subsequent studies.

The medium contained rHI, CLC, tropolone and ferric ammonium citrate, but did not contain Excyte, any other animal-derived proteins and even rHA.

A well developed serum-free medium should provide an environment for cells to grow quickly after being transferred from a serum-containing

Figure 5. Comparison of different lots and sources of recombinant human insulin (rHI) in animal-free medium for GS-NS0 Cell Line I. (a): Δ rHI Lot #1; $\square$ rHI Lot #2. Both lots were zinc salt of rHI made from yeast by the same vendor. (b): $\blacksquare$ Na-salt of rHI made from *E. coli*; $\blacktriangle$ Zn-salt of rHI made from yeast; $\square$ freshly prepared Na-salt of rHI; $\triangle$ freshly-prepared Zn-salt of rHI. All media contained 0.3 $\times$ CLC but no Excyte nor rHA. Graphs show growth profiles during multiple passages in shake-flasks for these cultures.

medium, without a prolonged period of adaptation, which would otherwise not only extend the overall process time but also increase the probability of selecting a variant or even a mutant. The above APF medium apparently satisfied this requirement, as it supported immediate, stable and good growth of NS0-WT ($\sim 2 \times 10^6$ vc/ml for 5 consecutive passages) upon seeding from 10% FBS growing culture or even vial-thaw without any adaptation (Figure 3). A similar conclusion was made for the GS-NS0 Cell Line II when transferred from 10% FBS culture (Figure 4), with IgG4 MAb production level also consistent for

multiple passages in this medium and very comparable to the SFII control cultures.

Lot-to-lot consistency and salt form-dependent variation of recombinant insulin

An important consideration in medium development is the consistency of constituent raw materials, especially those macromolecule or undefined components such as proteins. In the AF medium developed up to this stage, the only proteinaceous component was recombinant human insulin (rHI).

Figure 6. Consistent performance of zinc salt of recombinant human insulin (rHI) from different sources and ineffectiveness of sodium salt in animal-free medium for GS-NS0 Cell Line II. (a): $\circ$ Vendor 1; $\square$ Vendor 2; $\triangle$ Vendor 3. (b): $\triangle$ rHI zinc salt; $\square$ rHI sodium salt. All media contained $0.3 \times$ CLC but no Excyte nor rHA. The IgG4 titers shown were the day-7 results averaged from multiple passages. The 3rd passage cultures in (a) were not assayed for titers. Graphs show growth profiles during multiple passages in shake-flasks for these cultures.

Figure 7. Zinc chloride supplementation to cure rHI sodium salt for growth of GS-NS0 Cell Line I in animal-free medium. ● VCD by rHI zinc salt (from yeast); ■ VCD by rHI sodium salt (from *E. coli*); ▲ VCD by rHI sodium salt (from *E. coli*) plus zinc ions at 0.1x; ○ viability by rHI zinc salt (from yeast); □ viability by rHI sodium salt (from *E. coli*); △ viability by rHI sodium salt (from *E. coli*) plus zinc ions at 0.1x. All media contained 0.3x CLC but no Excyte nor rHA. Graphs show viable cell density and viability profiles during multiple passages in shake-flasks for these cultures.

Figure 8. Total replacement of rHI by zinc ions for growth of GS-NS0 Cell Line II in animal-free medium. □ rHI zinc salt; ○ without rHI; △ no rHI but added 1x zinc chloride. All media contained 0.3x CLC lipids, but no Excyte nor rHA. Graphs show viable cell density and viability profiles during multiple passages in shake-flasks for these cultures.

Two lots of rHI-zinc salt (made in yeast) from one vendor were compared, with no difference observed in supporting GS-NS0 Cell Line I growth (Figure 5a). However, it was surprising to find that when a sodium salt of rHI (made in *E. coli*) from another vendor was used as replacement, a significant difference in cell growth occurred (Figure 5b). The latter yielded less than 8×10^5 vc/ml

in growth, at least 50% lower than that by the zinc counterpart. This difference was apparently not due to the stability of rHI during storage as stock solution, since the freshly prepared sodium salt of rHI also supported poor growth (Figure 5b).

In the subsequent studies with Cell Line II, zinc salts of rHI from three yeast vendors again yielded very comparable growth and IgG4 production

Figure 9. Long-term growth and MAb production of GS-NS0 Cell Line III in animal- and protein-free medium. ▲ VCD; ● IgG₄ titer at Day-7. The medium contained 0.3× CLC lipids, but no Excyte nor rHA. Graphs show growth and Day-7 production during multiple passages in shake-flasks.

(Figure 6a). Similarly, dramatically lower growth was obtained when the sodium salt of rHI was used (Figure 6b), along with almost 3-fold lower IgG₄ titers.

Importance of zinc for culture growth and formulation of protein-free media

The unexpected poor performance of sodium salt versus zinc salt of rHI suggested certain important function(s) of zinc ions for NS0 cell growth. Indeed, with Cell Line I, it was observed that, similar to the case where no rHI was added, the culture viability with sodium-rHI declined dramatically to ~40% during passaging, but it was maintained at 70–80% level with zinc-rHI throughout (Figure 7). Encouragingly, supplementing zinc chloride to sodium-rHI restored the viability to the levels seen with zinc salt of rHI. Zinc ions reportedly can serve as anti-apoptotic agent and may enhance the survival of cells (Cotter and Rubeai 1995).

The positive effect of zinc supplementation to sodium-rHI led to the exploration of total replacement of rHI with increased concentrations of zinc ions. As shown in Figure 8, Cell Line II grew progressively more poorly without rHI and ionic zinc in AF medium, but with the mere supplementation of zinc ions (1×, 0.75×, 0.5×), the cells grew comparably to rHI-containing medium for multiple passages. Similar data were obtained for Cell Line I

(data not shown). The results also suggest that there was a wide window for effective zinc levels. The ionic zinc concentration was later optimized at 0.5× (data not shown). With the elimination of recombinant insulin, an animal-free (AF) and protein-free (PF) medium was formulated.

The ability to substitute for rHI with zinc ions appeared to be true across a variety of NS0 cell lines. Comparable peak growth and viabilities were obtained for NS0-WT cells irrespective whether rHI or ionic zinc was used in AF medium (data not shown). Also, for GS-NS0 Cell Line III, the zinc-supplemented protein-free AF medium supported $2\text{--}3 \times 10^6$ vc/ml of growth for over 20 passages with fairly consistent IgG₄ MAb production (Figure 9). For other cell lines such as Chinese Hamster Ovary (CHO), Gorfien et al. (1998) mentioned in their patent application for improved media that insulin could be replaced by zinc ions and transferrin by ferrous ions and ferrous chelate. During our preparation of this manuscript Wong et al. (2004a) reported that zinc was an effective insulin replacement for cell growth of hybridoma, myeloma and CHO-K1, but not CHO-IFN, although the conclusions were based on just one-passage data and the seeding densities were relatively high at $3\text{--}4 \times 10^5$ vc/ml. They also obtained comparable production by using either zinc or insulin for hybridoma cells.

Comparison of growth kinetics (Figure 10) revealed that NS0-WT cells utilized glucose more

Figure 10. Growth kinetics of NS0-WT cells in animal-free medium with rHI zinc salt or with zinc chloride. (a): ▲ VCD by rHI; △ VCD by 0.5× zinc chloride; ■ viability by rHI; □ viability by 0.5× zinc chloride. (b): ■ glucose by rHI; ▲ lactate by rHI; ● glutamine by rHI; □ glucose by 0.5× zinc chloride; △ lactate by 0.5× zinc chloride; ○ glutamine by 0.5× zinc chloride. All media contained 0.3× CLC lipids, but no Excyte nor rHA. Graphs show growth kinetics during a single passage in shake-flasks for these cultures.

efficiently and accumulated lactate to a lower extent when ionic zinc was used in place of rHI. It thus appeared that the insulin-like effect of zinc observed here related to glucose uptake/utilization. Zinc was shown to stimulate glucose transport in adipocytes (Izaki 1989; Ilouz et al. 2002).

Ionic zinc is involved in diverse cellular processes, both as a cofactor of numerous enzymes and as a regulatory factor. It was reported that zinc deprivation and excess affect gene expression in human THP-1 mononuclear cells (Cousins et al. 2003). But it remained unclear how exactly and how much zinc ions replaced rHI functions in cell culture. Ilouz et al. (2002) proposed that the *in vivo* insulin mimetic actions of zinc are mediated via direct inhibition of endogenous glycogen synthase kinase-3 β . Recently, by using DNA microarray to

study the effect of insulin and zinc on transcript profiles in hybridoma cultures, Wong et al. (2004b) detected that, at relatively high zinc level (5 mg/l ZnSO₄·7H₂O), genes coding for resistance to apoptosis were up-regulated, genes coding for transcription activation were over-expressed, and genes coding for activation of glucose uptake/metabolism were up-regulated. It was possible that although zinc might not interact at the same initial insulin binding site, the signaling pathways of both might converge at some point and result in overall similar effects on metabolism and cell division.

Applicability to cryopreservation

A desired medium should be able to cryopreserve the targeted cell lines and to revive the frozen cells

Figure 11. Effective cryopreservation and vial-thaw of NS0-WT cells in animal- and protein-free medium. $\blacktriangle$ VCD; $\triangle$ viability. The medium contained 0.3 $\times$ CLC lipids, but no Excyte nor rHA. Cryopreservation used the spent medium plus 10% DMSO. Vial-thaw was done by direct 1:20 dilution into the fresh medium. Graphs show viable cell density and viability profiles during multiple passages in shake-flasks for the culture prior and after cryopreservation.

without significantly losing culture viabilities. The developed animal- and protein-free medium was shown to meet this criterion satisfactorily. Figure 11 shows that, in the presence of 10% DMSO and without any serum or proteins, NS0-WT cells were frozen and revived successfully in this medium, maintaining 80–90% viabilities, and

growing for multiple passages to the same peak densities and same growth rates. Similar results were also obtained for the GS-NS0 cell lines (data not shown). In their simple serum-free freezing medium with 10% DMSO, Merten et al. (1995) observed that both Vero and BHK-21 cells could be cryopreserved and thawed without any impact

Figure 12. Growth of cholesterol-independent NS0-WT cells in Excyte-free SFII and animal-free chemically-defined media. $\circ$ SFII medium without Excyte; $\triangle$ animal-free chemically-defined medium (without rHA, rHI nor CLC, but with 1 $\times$ zinc chloride). Graphs show growth profiles during multiple passages in shake-flasks.

Figure 13. Growth performance of cholesterol-independent GS-NS0 Cell Line I in Excyte-free SFII and animal-free chemically-defined media. $\square$ SFII medium without Excyte; $\circ$ animal-free chemically-defined medium (without rHA, rHI nor CLC, but with $1\times$ zinc chloride). Graphs show growth profiles during multiple passages in shake-flasks.

on cell growth and viability, comparable to that achieved by DMEM containing 10% fetal calf serum and 10% DMSO as the freezing medium.

Development of chemically-defined medium for cholesterol-independent cell lines

NS0 cells are cholesterol-dependent but can be adapted to cholesterol-independence (Sato et al. 1987; Birch et al. 1994; Keen and Steward 1995). NS0 cells were derived from NS-1 cells that were deficient in 3-ketosteroid reductase activity thus unable to convert lathosterol to cholesterol (Sato et al. 1988). Keen and Steward (1995) found that a large portion of the NS0 population were able to adapt to cholesterol-independence, suggesting that this adaptation was likely due to the reactivation of the relevant gene rather than due to a revertant mutation. With a procedure similar to theirs, NS0-WT and GS-NS0 Cell Line I were thus gradually adapted to grow in the absence of lipids. The elimination of all lipids including cholesterol, together with the substitution of rHI with ionic zinc, enabled the formulation of a completely protein-free, chemically-defined medium for these cell lines. As shown in Figure 12, cholesterol-independent NS0-WT cells grew comparably well

at $\sim 2\times 10^6$ vc/ml for multiple passages in SFII medium (no Excyte) and in the animal-free, protein-free, and chemically-defined medium. Cholesterol-independent Cell Line I also grew immediately and consistently at $\sim 1.2\times 10^6$ in the protein-free defined medium for over 10 passages, although slightly lower than SFII (no Excyte) (Figure 13), which suggests a more stringent nutritional requirement by this recombinant transfectant. This cell line was then successfully cultured in 3-L spinners, achieving good IgG1 batch titers of 140 units/ml in 7 days (not shown).

Conclusion

By using literature search and component swapping strategies, bovine proteins commonly used in the serum-free media for NS0 cell culture were functionally replaced by recombinant insulin, tropolone and ferric ammonium citrate and non-animal-sourced lipid mixture to formulate animal-free media. Insulin was further eliminated by zinc supplementation to give rise to protein-free media. Adaptation of NS0 cells to cholesterol-independence enabled the development of completely defined media. These animal-free formulations supported adaptation-free growth and

MAb production that were comparable to the original animal protein-containing serum-free medium during multiple passages, for both wild-type host and various GS-NS0 constructs, and were also suitable for cryopreservation for these cells.

The above animal-free media were further optimized and successfully scaled-up to bioreactors with a fed-batch process developed, which is not within the scope of this publication.

Acknowledgements

The authors are very grateful to Barry Buckland for direction and encouragement, Lily Chu and her staff for recombinant NS0 cell lines, Craig Seamans, Sonal Munshi and Uma Balasubramanian for assistance in the development work, Roger Olewinski for Protein A HPLC assay, Amy Hsu for BIAcore quantitative assay, Peter Salmon for manuscript review, and many colleagues in Merck Bioprocess R&D for support to this effort.

References

- Allison D.W., Aboytes K.A., Fong D.K., Leugers S.L., Johnson T.K., Loke H.N. and Donahue L.M. 2004. Development and optimization of cell culture media. *BioProc. Int.* 2: 38–45.
- Barnes L.M., Bentley C.M. and Dickson A.J. 2000. Advances in animal cell recombinant protein production: GS-NS0 expression system. *Cytotechnology* 32: 109–123.
- Bebbington C.R., Renner G., Thompson S., King D., Abrams D. and Yarranton G.T. 1992. High level expression of a recombinant antibody from myeloma cells using a glutamine-synthetase gene as an amplifiable selectable marker. *Biotechnology* 10: 169–175.
- Birch J.R., Boraston R.C., Metcalfe H., Brown M.E., Bebbington C.R. and Field R.P. 1994. Selecting and designing cell lines for improved physiological characteristics. *Cytotechnology* 15: 11–16.
- Birch J.R. and Froud S.J. 1994. Mammalian cell culture systems for recombinant protein production. *Biologicals* 22: 127–133.
- Brown M.F., Renner G., Field R.P. and Hassell T. 1992. Process development for the production of recombinant antibodies using glutamine synthetase (GS system). *Cytotechnology* 9: 231–236.
- Buser C.W., Beaudet R., Soohoo N. and Pugh G.G. 1994. Development of serum-free media for engineered NS0 cell lines. In: Spier R.E., Griffiths J.B. and Berthold W. (eds), *Animal Cell Technology: Products of Today, Prospects of Tomorrow*. Butterworth-Heinemann, pp. 121–139.
- Castro P.M.L., Hayter P.M., Ison A.P. and Bull A.T. 1992. Application of a statistical design to the optimization of culture medium for recombinant interferon-gamma production by Chinese hamster ovary cells. *Appl. Microbiol. Biotechnol.* 38: 84–90.
- Cotter T.G. and Al-Rubeai M. 1995. Cell death (apoptosis) in cell culture systems and possible prevention using an anti-apoptotic gene or compound. *Trends Biotechnol.* 13: 150–155.
- Cousins R.J., Banchard R.K., Popp M.P., Liu L., Cao J., Moore J.B. and Green C.L. 2003. A global view of the selectivity of zinc deprivation and excess on genes expressed in human THP-1 mononuclear cells. *PNAS* 100: 6952–6957.
- Das R.C. and Morrow K.J. Jr. 2004. *Antibody Therapeutics: Product Development, Market Trends and Strategic Issues*. D&MD Publications, Westborough, MA.
- Dempsey J., Ruddock S., Osborne M., Ridley A., Stuart S. and Field R. 2003. Improved fermentation processes for NS0 cell lines expressing human antibodies and glutamine synthetase. *Biotechnol. Prog.* 19: 175–178.
- EMA 2002. Note for guidance on minimizing the risk of transmitting animal spongiform encephalopathies via human and veterinary medicinal products. EMA/410/01 Rev.
- FDA 2004. Tysabri (natalizumab). U.S. Food and Drug Administration web-site November 26, (2004) updated.
- Field R.P. 2003. Animal cell culture. US Patent #6593140, Lonza Group, AG, Assignee.
- Galbraith D.N. 2002. Transmissible spongiform encephalopathies and tissue cell culture. *Cytotechnology* 39: 117–124.
- Galfre G. and Milstein C. 1981. Preparation of monoclonal antibodies: strategies and procedure. In: Langone J.J. and Vunakis H.V. (eds), *Methods Enzym.* Academic Press, 73: 3–46.
- Gambhir A., Zhang C., Europa A. and Hu W.S. 1999. Analysis of the use of fortified medium in continuous culture of mammalian cells. *Cytotechnology* 31: 243–254.
- Gorfien S., Paul B., Walowitz J., Keem R., Biddle W. and Jayme D. 2000. Growth of NS0 cells in protein-free, chemically-defined medium. *Biotechnol. Prog.* 16: 682–687.
- Gorfien S.F., Fike R.M., Dzimian J.L., Godwin J.P., Price P.J., Epstein D.A., Gruber D. and McClure C. 1998. Suspension culture of mammalian cells in medium containing polycationic or -anionic compound. Patent application WO9808934.
- Hellman K.B., Honstead J.P. and Vincent C.K. 1996. Adventitious agents from animal-derived raw materials and production systems. *Develop. Biol. Standard.* 88: 231–234.
- Ilouz R., Kaidanovich O., Gurwitz D. and Eldar-Finkelman H. 2002. Inhibition of glycogen synthase kinase-3 β by bivalent zinc ions: insight into the insulin-mimetic action of zinc. *Biochem. Biophys. Res. Comm.* 295: 102–106.
- Izaki O. 1989. IIb group metal ions (Zn²⁺, Cd²⁺, Hg²⁺) stimulate glucose transport activity by post-insulin receptor kinase mechanism in rat adipocytes. *J. Biol. Chem.* 264: 16118–16222.
- Jayme D.W. 1991. Nutrient optimization for high density biological production applications. *Cytotechnology* 5: 15–30.
- Jayme D.W. 1999. An animal origin perspective of common constituents of serum-free medium formulations. *Develop. Biol. Standard.* 99: 181–187.
- Keen K.J. and Hale C. 1996. The use of serum-free medium for the production of functionally active humanized monoclonal antibody from NS0 mouse myeloma cells engineered using glutamine synthetase as a selectable marker. *Cytotechnology* 18: 207–217.

- Keen M.J. and Steward T.W. 1995. Adaptation of cholesterol-requiring NS0 mouse myeloma cells to high density growth in a fully defined protein-free and cholesterol-free culture medium. *Cytotechnology* 17: 203–211.
- Kohler G. and Milstein C. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* 6: 511–519.
- Kovar J. and Franek C. 1987. Iron compounds at high concentrations enable mouse hybridoma and myeloma cells to grow in protein-free medium. *Eur. Congr. Biotechnol.* 3: 614–617.
- Mainwaring D.O., Maxwell A., Rendall M.H., Blewett J.M., Racher A.J. and Wayte J.R.T. 2002. Development of improved chemically-defined mammalian cell culture fermentation processes. Poster presentation at 224th ACS National Meeting, Boston, MA.
- Metcalfe H., Field R.P. and Froud S.J. 1994. The use of 2-hydroxy-2,4,6-cycloheptarin-1-one (tropolone) as a replacement for transferrin. In: Spier R.E., Griffiths J.B. and Berthold W. (eds), *Animal Cell Technology: Products of Today, Prospects of Tomorrow*. Butterworth-Heinemann, pp. 88–90.
- Merten O.W. 1999. Safety issues of animal products used in serum-free media. *Develop. Biol. Standard.* 99: 167–180.
- Merten O.W. 2002. Virus contaminations of cell cultures – a biotechnological view. *Cytotechnology* 39: 91–116.
- Merten O.W., Petres S. and Couve E. 1995. A simple serum-free freezing medium for serum-free cultured cells. *Biologicals* 23: 185–189.
- Mullett W.M., Lai E.P.C. and Yeung J.M. 2000. Surface plasmon resonance-based immunoassays. *Methods* 22: 77–91.
- Robinson D.K., Distefano D.J., Gould S.L., Cuca G., Seamans T.C., Benincasa D., Munshi S., Chan C.P., Lee D.K., Stanford-Hollis J., Hollis G.F., Jain D., Ramasubramanyan K., Mark G.E. and Silberklang M. 1995. Production of engineered antibodies in myeloma and hybridoma cells – enhancements in gene expression and process design. In: *Antibody Engineering*. ACS Symposium Series 604 pp.1–14.
- Robwer R.G. 1996. Analysis of risk to biomedical products developed from animal sources (with special emphasis on the transmissible spongiform encephalopathies, TSEs). *Develop. Biol. Standard.* 88: 247–256.
- Sato J.D., Kawamoto T. and Okamoto T. 1987. Cholesterol requirement of P3-X63-Ag8 mouse myeloma cells for growth *in vitro*. *J. Exp. Med.* 165: 1761–1766.
- Sato J.D., Cao H.T., Kayada Y., Cabot M.C., Sato G.H., Okamoto T. and Welsh C.J. 1988. Effect of proximate cholesterol precursors and steroid hormones on mouse myeloma growth in serum-free medium. *In Vitro* 24: 1223–1228.
- Schmelzer A.E., Weymer C.L. and Varma A. 2004. Optimized fed-batch bioreactors for NS0 antibody production. Presentation at 227th ACS National Meeting, Anaheim, CA.
- Talley D., Cutak B., Rathbone E., Al-Kolla T., Allison D., Blasberg J., Kao K. and Caple M. 2003. SyntheChoTM synthetic cholesterol for cholesterol dependent cell culture – development of non-animal derived chemically defined NS0 medium. *Sigma Technical Bulletin*.
- Tsao Y.S., Gould S.L. and Robinson D.K. 2000. Cell culture media. In: Miller W. et al. (eds), *Encyclopedia of Animal and Plant Cell Technology*. J. Wiley & Sons, pp.35–31.
- Varma A., Schmelzer A.E., White S., Patel M., DiGirolamo C. and Case S. 2003. Enhanced antibody production yields from NS0 cells. Presentation at the 8th Watersides Conference, San Francisco, CA.
- Whitford W., Barnett B. and Pence B. 2004. HyQ@CDM4NS0TM: the next generation CD NS0 culture medium. Poster presentation at IBC/BioProcess International Conference & Exhibition, Boston, MA.
- Wong V.T., Ho K.W. and Yap G.S. 2004a. Evaluation of insulin-mimetic trace metals as insulin replacements in mammalian cell cultures. *Cytotechnology* 45: 107–115.
- Wong V., Nissom P.M., Sim S.L., Yeo J. and Yap M. 2004b. Correlating the productivity and transcriptional profile of hybridoma cells. Presentation at 227th ACS National Meeting, Anaheim, CA.
- Xie L. and Wang D.I.C. 1994. Application of improved stoichiometric model in medium design and fed-batch cultivation of animal cells in bioreactor. *Cytotechnology* 15: 17–29.
- Xie L. and Wang D.I.C. 1997. Integrated approaches to the design of media and feeding strategies for fed-batch cultures of animal cells. *Trends Biotechnol.* 15: 109–113.
- Zhou W., Chen C.C., Buckland B. and Aunins J. 1997. Fed-batch culture of recombinant NS0 myeloma cells with high monoclonal antibody production. *Biotechnol. Bioeng.* 55: 783–792.